SUMMARY

Monitoring of the biochemical reactions, understanding of the catalytic mode of the enzymes and improving the efficacy of currently available anticancer drugs are some of the contemporary areas of bioorganic and medicinal research. Sensing of the biological entities like adenosine diphosphate (ADP) and adenosine triphosphate (ATP) is one of the most popular tools to visualize the complicated biological processes due to the fact that most of the biochemical reactions are coupled with ADP – ATP inter-conversions. Likewise, the enzyme models provide a simplified alternative route to the complex enzymatic reactions and give fruitful information about the mechanism of enzyme catalyzed reactions. In order to achieve these targets, availability of intuitively and intelligently designed molecules is a basic requirement. In certain cases, the serendipitous discoveries proved equally important and resulted into the development of new drugs as well as the molecular probes for studying various biochemical processes. However, despite the wide range of studies for the design and synthesis of new molecules for their use as biological probes as well as the enzyme models and even the drugs; a number of bottleneck including selectivity, solubility, robustness, efficiency, economical synthesis, side effects etc are still there.

Hypothesizing that the inherent selectivity of small natural products could be employed for procuring the substrate specific molecules; here, we used acridine, amino alcohol and amino acids for constructing desirable compounds. Acridine was used as the template and derivatized with amino alcohol and amino acids. Advantage was made of the fluorescent nature of the acridine. The new molecules were studied for their ADP/ATP selectivity, for the modelling of aldolase catalysis and in the development of new anti-cancer agents. Review of literature on the basis of ADP/ATP selective probes, retero-aldolase models and acridine based anticancer drugs has been given in chapter 1. For the sake of presentation, the research work has been divided into chapter 2-4.

CHAPTER-2: ADP SELECTIVE PROBE AND STUDY OF GLUCOSE METABOLISM

2A. SYNTHESIS

With an idea to increase the fluorescence intensity and quantum yield of the compound by introducing two acridine moieties in the molecule in comparison to one of our previous ATP selective probe\(^1\) led us to synthesize dimeric type of compounds 2
and 3. Synthesis of acridine from anthranilic acid and 2-chlorobenzene was followed by its reaction with epichlorohydrin in presence of NaH to procure compound 1. Further reaction of compound 1 with piperazine and 2-[2-(2-aminoethoxy)ethoxy]ethylamine under microwave irradiation at 140 °C in CH₂Cl₂ provided compound 2 and 3 respectively (Scheme 1).

**Scheme 1**

**2B. ADP INTERACTION STUDY**

**Fluorescence and mass spectral studies**

Fluorescence spectrum of solution of compound 2 (10 µM) in DMSO – HEPES buffer (1:4) exhibited strong emission at 427 nm (Φ = 0.63) when excited at 250 nm. Incremental (12 µL) addition of ADP (0.5 ng in 12 µL HEPES buffer, 0.1 µM) to the solution of compound 2 (1.2 ml in DMSO-HEPES buffer, 1:4) decreased the fluorescence intensity of the solution (trace A to trace B, Figure 1) after the addition of 360 µL of ADP. No further change in fluorescence intensity was observed when more ADP was added. However, addition of 360 µL of ATP (same concentration as for ADP) to the solution of compound 2 made almost quenching of the fluorescence (trace A to
trace C, Figure 1). It means compound 2 is responding to both ATP and ADP and exhibited non-selectivity between the two nucleotides.

Figure 1. Change in fluorescence intensity of solution of compound 2 (10\(^{-5}\) M) in DMSO:HEPES buffer (1:4), pH 7.2 (trace A) on addition of ADP (trace B), and addition of ATP (trace C). Excitation wavelength 250 nm.

Fluorescence spectrum of solution of compound 3 (10 µM) in DMSO – HEPES buffer (1:4) also showed strong emission band at 430 nm (Φ = 0.68) when excited at 250 nm. Stepwise (12 µL) addition of ADP (0.5 ng in 12 µL HEPES buffer, 0.1 µM) to the solution of compound 3 (1.2 ml in DMSO-HEPES buffer, 1:4) (final concentration of ADP in 1.2 ml solution was 0.001 µM) resulted into decrease in fluorescence intensity of the solution until complete quenching of fluorescence was taken place when 153 ng ADP (addition of 360 µL of ADP, 1 µM) was added to the solution (Figure 2A). However, no change in fluorescence of solution of compound 3 was observed in presence of even 300 ng (in 12 µL HEPES buffer) ATP (45 µM), AMP (72 µM) and other nucleotides (GTP, GDP, CTP, CDP, UTP, UDP) indicating the selectivity of compound 3 for ADP (Figure 2B).

Electrospray ionization high resolution mass spectrum (ESI-HRMS) of solution of compound 3 with ADP also showed formation of 3.ADP complex (Figure 2C). Hence, a fluorescent probe, workable in aqueous medium and showing considerable selectivity for ADP is identified which was proved helpful in quantification of ADP.
during a biochemical reaction as well as in biological samples. Besides a recent report by Feng et al about a mononuclear zinc complex appended with two anthracene groups as highly selective ADP sensor, compound 3 is amongst a very few ADP selective probes reported so far. In order to utilize the results of these experiments for monitoring of ATP – ADP coupled biochemical reactions, a curve showing change in fluorescence intensity of solution of compound 3 as a function of ADP concentration was standardized (Figure 2D).

Figure 2. A) Decrease in fluorescence intensity of solution of compound 3 (10^5 M) in DMSO:HEPES buffer (1:4), pH 7.2 on stepwise addition of ADP. Excitation wavelength 250 nm; B) Fluorescence change of solution of compound 3 in DMSO:HEPES buffer (1:4, pH 7.2) in presence of other nucleotides (45 µM) showing selective and competitive binding of compound 3 with ADP; C) HRMS of solution of compound 3 and ADP showing formation of 3+ADP complex with m/z 1078.3378 (calcd m/z 1078.3471); D) Normalized curve for change in fluorescence intensity of solution of compound 3 as a function of ADP concentration (black line). Red line indicates actual position of data points.

2C. QUANTIFICATION OF ATP/ADP IN GLUCOSE METABOLISM AND BREAKDOWN OF PYRUVATE IN MITOCHONDRIA

1. Monitoring of glucose metabolism (Phase I)

   (i) Phosphorylation of glucose
Solution of D-glucose (50 µM), hexokinase (80 units), Mg$^{2+}$ (50 µM) and compound 3 (10 µM in DMSO) in HEPES buffer (2 ml) showed usual fluorescence intensity of compound 3 (trace A, Figure 3A) indicating no effect of glucose and hexokinase on the fluorescence behaviour of the compound. The moment 100 µL ATP (4 ng ATP in 100 µL HEPES buffer; 0.0036 µM in 2 ml final solution) was added to this solution, the fluorescence intensity started decreasing and becomes constant at 8000 A.U. (trace B, Figure 3A). As calculated from the standard curve (Figure 2D), the amount of ADP in the solution is ~3.1 ng (0.0036 µM) which indicates that all the ATP in the solution has been changed to ADP. With same amount of reactants (as used above), kinetic analysis of this enzymatic reaction was studied by varying the concentration of hexokinase and it was found to be first order (Figure 3B).

![Figure 3](image_url)

**Figure 3.** A) Change in fluorescence from A to B during the reaction of first step of glucose metabolism; B) Intial rate as a function of amount of hexokinase; C) Lineweaver-Burk plot; D) a part of HRMS of reaction mixture of first step of glucose metabolism.

The Lineweaver-Burk plot (Figure 3C) gave Michaelis-Menten constant ($K_m$) 125 µM corresponding to the reported one 160 µM. HRMS of the reaction mixture of this step of glucose metabolism (Figure 3D) did not show peak due to mass of ATP.
while the peak at m/z 261.0355 corresponding to mass of phosphorylated glucose (calcd m/z 261.0370 [M+H]+) is quite intense.

(ii) **Phosphorylation of fructose 6-phosphate**

Similar to the monitoring of above reaction, phosphorylation of fructose-6-phosphate was performed.

(iii) **Formation of 3-phosphoglycerate**

During the dephosphorylation steps of glucose metabolism, the measurement of increase in fluorescence intensity of the non-fluorescent solution of compound 3 (in presence of ADP) also gave quantitative information about ADP consumed and consequent formation of ATP.

(iv) **Conversion of phosphoenolpyruvate to pyruvate**

For monitoring the last step of payoff phase of glycolysis viz. conversion of phosphoenolpyruvate to pyruvate, solution of phosphoenolpyruvate (50 µM), compound 3 (10 µM) and ADP (50 ng ADP in 100 µL HEPES buffer; 0.058 µM in 2 ml assay solution) in 2 ml HEPES buffer (pH 7.2) was prepared. After the addition of pyruvate kinase (80 units) to the reaction mixture, the solution slowly turned fluorescent and within 2 min, it gained same fluorescence as observed for solution of compound 3 alone. This change in fluorescence intensity of the solution clearly indicated the consumption of all the ADP.

2. **Quantification of ADP in mitochondria**

Solution of 20 µL of compound 3 (DMSO) and 10 mg mitochondrial mass in 100 µL resuspended buffer (extracted from pig liver and refined as per the reported procedure) in 1 ml final solution in HEPES buffer (compound 3 is 10 µM) showed fluorescence as per trace B of Figure 4. This decrease in fluorescence intensity of otherwise fluorescent solution of compound 3 (trace A, Figure 4) was assumed to be due to the presence of ADP in mitochondrial system because almost all the other components associated with mitochondria were removed. As per the standard curve (Figure 2D), ~50 ng of ADP seems to be there in the tested sample of mitochondria.
Summary

Figure 4. Change in fluorescence of solution of compound 3 from trace A to trace B when mitochondrial solution was added.

In conclusion, using fluorescent probe 3, all those steps of glucose metabolism which involve either consumption of ADP or generation of ADP were monitored. Pyruvate breakdown in mitochondria and quantification of ADP in mitochondria were also monitored.

CHAPTER-3: RETRO-ALDOLASE MODEL FOR THE CONVERSION OF D-FRUCTOSE TO GLYCERALDEHYDE AND DIHYDROXYACETONE

3A. DESIGN AND SYNTHESIS OF RETROALDOLASE MODELS

Inspired by the working of retroaldolase, here, we demonstrated the optimization of a tripeptide appended acridine as a retroaldolase model for D-fructose substrate. Lys and Tyr were incorporated on a rigid platform of acridine and the stereochemistry at Cα and the spacing between these two amino acids was adjusted to make the molecule capable of capturing D-fructose and catalyzing further transformations. The acridines 4-6 (Chart 1), derivatized at C-4 with Tyr-Lys, Tyr-Gly-Lys, Tyr-Gly-Gly-Lys and Lys-Gly-Tyr peptide were designed and synthesized.
Chart 1. Library of model compounds. Numbering 1 and 2 is only to represent the asymmetric carbons.

Reaction of anthranilic acid and 2-chlorobenzoic acid in iso-amyl alcohol in the presence of \( \text{K}_2\text{CO}_3 \) and CuO provided 9-oxo-9, 10-dihydroacridine-4-carboxylic acid. Designed compound were synthesized from the reaction of 9-oxo-9, 10-dihydroacridine-4-carboxylic acid with tyrosine methyl ester, glycine methyl ester, H-Lys(Z)methyl ester and phenyl alanine methyl ester in the presence of ethyl chloroformate, triethyl amine, LiOH and Pd-C-H\(_2\) as discussed in thesis.

3B. REACTION OF MODEL COMPOUNDS WITH FRUCTOSE

Mass and NMR spectral studies

Reactions of all the compounds with D-fructose (1:1) were performed in DMSO – H\(_2\)O (3:7, v/v) at 25 – 27°C and pH 2 – 12. The reactions were monitored by taking out the aliquots of the reaction mixture at 5 min intervals and recording the high resolution mass spectra (ESI-HRMS). Schiff base formation was observed in all the reactions after 5 min of stirring at pH 6.0 – 6.5 (Figure 1A, Figure 2, 3). The peak at \( m/z \) 707.2925, 764.3134 and 821.3350 in the HRMS of the reaction mixtures of compounds 4, 5, and 6, respectively corresponded to the \( m/z \) of their respective Schiff base ions (calcd \( m/z \) 764.3137, 707.2927, 821.3352; [M]^+). In contrast to the normal appearance of protonated adduct in +ve ESI mode [M+H]^+, here, all the Schiff bases seem to exist in the charged imine form [M]^+, no peak was detected in the –ve ESI mode. There was
no further change in any one of these reactions. The pH of the reaction mixture was varied between 6.0–3.0 and 6.0–10.0. Characteristically, in the reaction mixture of compound 5d, the peak at \( m/z \) 764.3134 (due to Schiff base 8) was almost disappeared (Figure 1B) and a prominent peak at \( m/z \) 91.0311 was visible (Figure 1C) when the reaction mixture was stirred at pH 7.5 – 8.0 for 30 min.

**Figure 1.** HRMS of the reaction mixture of compound 5d and D-fructose after (A) 5 min of stirring, (B) 15 min of stirring, (C) part of spectrum B is expanded to show the peak at \( m/z \) 91.0311. Low intensity peaks at 79 and 101 correspond to protonated DMSO and sodiated DMSO, respectively.

**Figure 2.** Mass spectrum of reaction mixture of compound 4 and D-fructose. Peak at \( m/z \) 707.2925 corresponds to mass of Schiff base ions (calcld \( m/z \) 707.2923, [M]+). Peaks at \( m/z \) 181.0703 and 545.2391 correspond to \( m/z \) of fructose and compound 4 ions, respectively.
Figure 3. Mass spectrum of reaction mixture of compound 6 and D-fructose. Peak at m/z 821.3350 corresponds to mass of Schiff base ions (calcd m/z 821.3352, [M]+). Peaks at m/z 181.0712 and 659.2824 correspond to m/z of fructose and compound 6 ions, respectively.

Figure 4. (A) LC chromatogram of thick oil isolated from the reaction of compound 5d and D-fructose. (B) Overlapped LC chromatogram of commercial sample of L-glyceraldehyde, D-glyceraldehyde and dihydroxyacetone. Chirobiotic® T 10 µm chiral HPLC column and ACN – H₂O (1:1) charged with 0.1% formic acid was the eluent.

Figure 5. (A) LC chromatogram of thick oil obtained after work up of reaction mixture of compound 5d and D-fructose. LC chromatogram of commercial samples of: (B) D-glyceraldehyde, (C) Dihydroxyacetone and (D) L-glyceraldehyde.

Due to the decrease in intensity of the fructose ions mass peak, it was anticipated that the peak at m/z 91.0311 might have originated due to the fragmentation of fructose. The peak at m/z 91.0311 was assigned to glyceraldehyde/dihydroxyacetone (calcd m/z 91.0390, [M+H]+). None of the other reactions underwent such type of changes as observed in the reaction of 5d. The oily product (55%) obtained from the reaction of 5d
and D-fructose showed two peaks in the LC chromatogram (Figure 4A), each having $m/z$ 91.0380. Comparison with the authentic samples (Figure 4B, Figure 5) confirmed the identity of one of the two LC peaks as D-glyceraldehyde and the second as dihydroxyacetone. 30% Fructose was also recovered along with the isolation of 65% 5d.

3C. REACTION KINETICS

Kinetics of the reaction has discussed in detail in the thesis.

In conclusion, the results of these experiments have unambiguously ensured that 5d is catalyzing the cleavage of D-fructose to D-glyceraldehyde and dihydroxyacetone.

CHAPTER 4: ACRIDINE BASED ANTI-CANCER AGENTS

4A. SYNTHESIS OF AMINO ACID APPENDED ACRIDINES

Amino acid appended acridines have emerged as a new category of anti-cancer drugs. Anti-proliferative activity of some of the molecules of this category has been documented. Following Lipinski’s rule of ‘5’, we planned to introduce amino acids on acridine template. Treatment of acridone 1 with L-valine methyl ester hydrochloride in the presence of triethyl amine and ethyl chloroformate provided compound 2.
Similarly, treatment of acridone 1 with L-tyrosine methyl ester hydrochloride provided compound 3 (Scheme 1). Keeping in mind the hydrophilicity/hydrophobicity factor, amino acid chain of compound 4 was further extended by introducing proline and glycine. As depicted in scheme 2, compound 4 was treated with L-proline methyl ester hydrochloride to procure compound 5. Ester hydrolysis of compound 5 and subsequent treatment with glycine methyl ester hydrochloride provided compound 7 which on treatment with LiOH gave compound 8. Likewise, the sequential incorporation of glycine and proline in compound 4 provided compound 12. All the compounds were characterized with the help of various spectroscopic techniques.

Reaction conditions:
(i) Et3N, EtOOCocl, L-Proline methyl ester hydrochloride
(ii) Et3N, EtOOCocl, Glycine methyl ester hydrochloride
(iii) LiOH/Acetonitrile/water (2:1)
(iv) Et3N, EtOOCocl, L-Proline methyl ester hydrochloride
(v) Et3N, EtOOCocl, Glycine methyl ester hydrochloride

Scheme 2
4B. **ANTIPROLIFERATIVE STUDY**

Compounds 2, 3, 4, 8, 9 and 12 at 0.5 μM to 50 μM concentration were screened through MTT assay for their antiproliferative activity. The IC$_{50}$ of these compounds was in the range of 14-20 μM. Cell cycle analysis has clearly shown that the compounds under present investigation arrest C6 cells in G$_0$/G$_1$ phase of the cell cycle. In comparison to the other compounds, 8, 9 and 12 resulted in statistically significant arrest of the cells in G$_0$/G$_1$ phase of the cell cycle and hence these three compounds were subjected to further investigation.

Phase contrast imaging of cells indicated that concentrations above 10 μM were toxic (Figure 1 and 2) which resulted in distorted cell morphology so 10 μM concentration was selected for further studies. Cells treated with 10 μM concentration of 8, 9 and 12 showed significant decrease in rate of proliferation of C6 cells as compared to control cultures (Figure 3A).

![Image of phase contrast images](image)

**Figure 1.** Phase contrast images of Cells treated with 8, 9, 12 at 0.5 μM, 1 μM, 5 μM, 10 μM, 20 μM, 30 μM, 40 μM and 50 μM.
Figure 2. α-Tubulin immunostaining showing distorted cell morphology treated with 20 μM concentration of 9, 8 and 12 as compared to control.

Flow cytometry based detailed cell cycle analysis showed significant decrease in the cell number in G2/M and S phase after treatment with compounds 8, 9 and 12 in comparison to the control (Figure 3C), thus showing reduction in their proliferation. On the other hand, a significant increase was observed in G0/G1 population of cells after exposing to test compounds. These observations suggest that the compounds 8, 9 and 12 arrested the cancer cell population in the resting phase i.e. G0/G1 phase of the cell cycle, like the normal cells, and thus may prove to be the potential anti-proliferative compounds.

Figure 3. (A). Phase contrast micrographs of C6 glioma – control and treated with 8, 9, 12. (B). MTT assay showing decrease in the cell number upon treatment with 8, 9, 12. (C). Histogram representing distribution of cells in G0/G1, S and G2/M phase of cell cycle analyzed by PI using flow cytometer. Values are presented as mean ± SEM of at least three independent experiments. ‘*’ (P<0.05), represent statistical significant difference between control and treated groups.
Phase contrast micrographs and confocal images of α-tubulin immunostained cells also showed reduced cell number and well differentiated morphology with long stellate processes and small cell body in the treated cultures. GFAP is an astrocyte specific intermediate filament (IF) protein marker and many CNS pathologies such as trauma and tumor have been shown to cause astrogliosis which is characterized by enhanced GFAP expression and astrocytic proliferation. The current data shows that treatment of C6 cells with 8, 9 and 12 was associated with down regulation of GFAP expression (Figure 4B and 4A). Based on these observations, it may be suggested that these test compounds have the potential to suppress the proliferation as well as the reactive astrogliosis seen in C6 glioma tumor cells.

**Figure 4.** Confocal images of C6 glioma cells showing α-Tubulin (A) and GFAP (B) expression in control (Ia), 9 (Ib), 8 (Ic) and 12 (Id) treated cells. Right panel of α-tubulin (A IIa-d) is enlarged view of single cell corresponding to images in left panel (A Ia-d). Similarly, the right panel of GFAP (B IIa-d) is the enlarged view of single cell corresponding to images in the left panel (B Ia-d).
To further elucidate the senescence inducing potential of these synthetic compounds, the expression of senescence markers such as heat shock family protein members-mortalin and HSP70 was studied (Figure 5). Immortal cells are reported to have mortalin expression in perinuclear region, whereas, normal cells show pancytoplasmic distribution of this protein. In control C6 glioblastoma cells, perinuclear expression of mortalin was observed which got translocated to pancytoplasmic locations and nucleus after treatment with 8, 9 and 12. Interestingly, the nuclear expression of mortalin was also upregulated (Figure 5A). This perinuclear to pancytoplasmic translocation is reported to be related with induction of senescence, whereas, a recent report suggested that nuclear mortalin play an important role in differentiation induction via retinoid receptor interactions. These observations are also supported by previous reports from our laboratory on anticancer and differentiation inducing activities of some natural products.

**Figure 5.** Confocal images of C6 glioma cells showing Mortalin (A) and HSP70 (B) expression in control (Ia), 9 (Ib), 8 (Ic) and 12 (Id) treated cells. Right panel of Mortalin (A IIa-d) is enlarged view of single cell corresponding to images in the left panel (A Ia-d). Similarly, the right panel of HSP70 (B IIa-d) is the enlarged view of single cell corresponding to images in the left panel (B Ia-d).
This upregulated expression of mortalin (Figure 5A) was also followed by elevated expression of mitochondrial stress response protein HSP70. HSP70 is an ATP-dependent housekeeping gene, which is highly involved in glial cell differentiation and neurite outgrowth. There was significant increase in HSP70 expression in cells treated with 10 μg/ml concentration of 8, 9 and 12 (Figure 5B) which further supports the senescence and differentiation inducing potential of these compounds.

In conclusion, the preliminary investigation of a series of amino acid appended acridines revealed considerable potential of these compounds as anticancer agents. Compounds exhibited significant antiproliferative activity against C6 glioblastoma cell line. This may be possibly attributed to arresting of cells in G0/G1 phase of the cell cycle. The compounds were also seen to upregulate mortalin and HSP70 expression thus indicating their senescence inducing potential.